Localization of the Peptide Binding Domain of the NK-1 Tachykinin Receptor Using Photoreactive Analogues of Substance P

N. D. BOYD,[a] R. KAGE,[a] J. J. DUMAS,[a] S. C. SILBERMAN,[a]
J. E. KRAUSE,[b] AND S. E. LEEMAN[a]

[a]Department of Pharmacology and Experimental Therapeutics
Boston University School of Medicine
Boston, Massachusetts 02118

[b]Department of Anatomy and Neurobiology
Washington University School of Medicine
St. Louis, Missouri 63110

Substance P is a member of a family of structurally related neuropeptides known collectively as the tachykinins. The tachykinin peptides are involved in diverse biological functions both in the central nervous system and the periphery. In contrast to the smaller classical transmitters such as acetylcholine where functional diversity results from the interaction of a single transmitter with multiple receptor subtypes, a different strategy has evolved for the tachykinin peptide family. For each of the tachykinin peptides a distinct but homologous receptor exists;[1] to date there is no definitive evidence for multiple receptor subtypes. The specific receptor for substance P (SP) has been termed the NK-1 receptor. This receptor binds substance P with an affinity that is several orders of magnitude greater than for all other members of the tachykinin peptide family.[2] To determine the basis for this peptide selectivity, we are attempting to map the residues of the NK-1 receptor that are involved directly in substance P recognition and subsequent transmembrane signaling by this receptor.

For these studies we developed a new approach to photoaffinity labeling that relies on incorporating the photoactivatable benzophenone group at different locations within the 11-amino acid sequence of substance P.[3,4] The use of the benzophenone group as a photolabel has several advantages over more conventional photolabels such as the azide group. These advantages include a high efficiency of photoincorporation and the chemical stability of the benzophenone group under peptide synthesis conditions.[4,5] When photoactivated by exposure to 350 nm light, the carbonyl group of the benzophenone moiety undergoes a n-π^* transition, forming a triplet biradical oxygen. The first and rate determining step involves hydrogen abstraction from a C-H bond followed by formation of a stable C-C bond between the peptide probe and an amino acid residue present at the peptide binding site of the receptor[6,7] (FIG. 1).

p-Benzoyl-L-phenylalanine (Bpa) is an unnatural amino acid that contains a benzophenone side chain. Two photoaffinity peptides were used in this study: (Bpa[8])-SP and (Bpa[4])-SP (FIG. 2). Substitution in position 8 was chosen because studies in our own laboratory and in several others have shown that this position can accommodate a range of aliphatic and aromatic amino acids.[8] In addition, substitu-
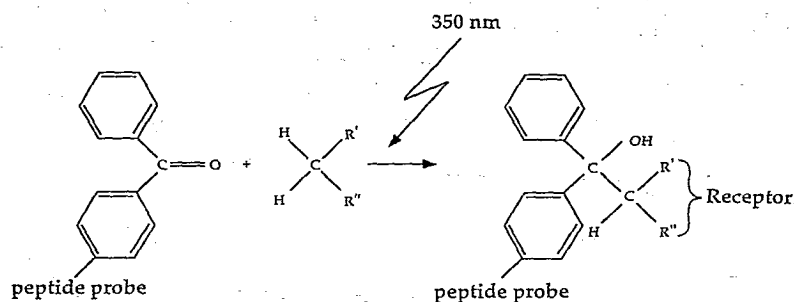
FIGURE 1. Photochemistry of benzophenone.

tion in position 8 places the photoreactive benzophenone group in the conserved C-terminal sequence that defines the tachykinin peptide family and is necessary for interaction and activation of the receptors of these peptides. Substitution in position 4 was selected because it places the photoreactive group in the middle of the divergent N-terminal sequence of substance P. Although the N-terminal sequence is not essential for binding, interactions between residues of this region and the NK-1 receptor are necessary for binding with high subnanomolar affinity and thus contribute to the selectivity of the NK-1 receptor for substance P.[2] Radioiodinated deriva-
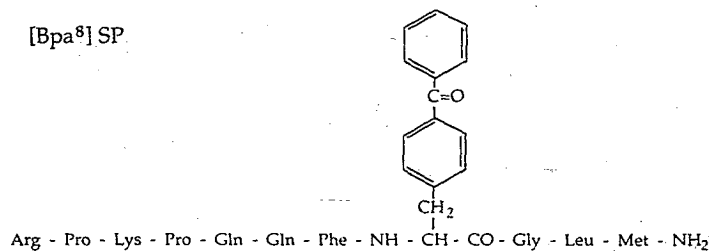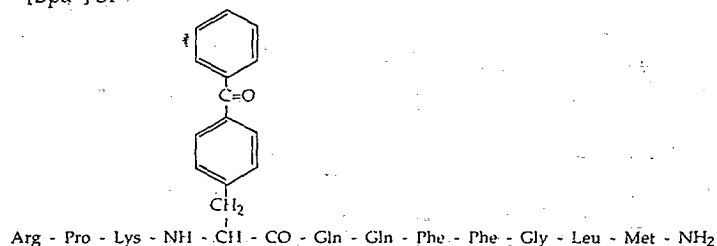

FIGURE 2. Chemical structure of substance P (SP) analogues in which the photoreactive amino acid, p-benzoyl-L-phenylalanine (Bpa) has been incorporated in position 4, (Bpa[4])-SP (lower panel), and position 8, (Bpa[8])-SP (upper panel).

tives of each peptide were prepared using [^{125}I]labeled Bolton Hunter reagent which acylates the ε-amino group of the lysine residue in position 3.[4]

Preliminary binding and functional studies established that both these substance P analogues are agonists that bind to NK-1 receptors with an affinity similar to that of the parent peptide, substance P.[4] However, unlike substance P, both of the Bpa-containing substance P analogues become covalently attached to the receptor upon irradiation with ultraviolet (UV) light. The photoaffinity probes have been used to detect and characterize NK-1 receptors in a variety of tissues.[2] However, for the mapping studies reviewed here, we used as a source of NK-1 receptors a stably transfected Chinese hamster ovary cell line that expresses about 5×10^6 rat NK-1 receptors per cell. In the photolabeling experiment shown in FIGURE 3, NK-1 receptors expressed in CHO cells were photolabeled with [^{125}I]-(Bpa^8)-SP or [^{125}I]-(Bpa^4)-SP. The photolabeled receptors were resolved by SDS-PAGE, and the
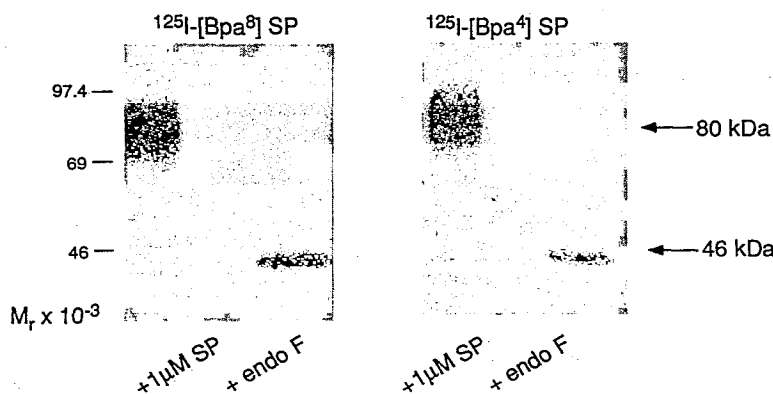

FIGURE 3. Photoaffinity labeling of NK-1 receptors expressed in Chinese hamster ovary (CHO) cells by [^{125}I]-(Bpa^4)-SP and [^{125}I]-(Bpa^8)-SP. Transfected CHO cells expressing the NK-1 receptors were equilibrated with [^{125}I]-(Bpa^4)-SP and with [^{125}I]-(Bpa^8)-SP in the absence (−) and presence (+) of 1 μM of substance P and photolyzed at 4 °C for 10 min with 350 nm light. Membranes prepared from the photolabeled cells were subjected to SDS-PAGE, and the labeled bands were visualized by autoradiography. Photolabeled membranes were also solubilized and treated with endoglycosidase F prior to analysis by SDS-PAGE/autoradiography.

position of the labeled receptor detected by autoradiography. The photolabeled receptors are visualized as a broad radioactive band centered at $M_r = 80,000$. Photolabeling by both probes is highly specific because no radiolabeled bands are detectable when photolabeling is conducted in the presence of an excess of unlabeled substance P. Photolabeling in each case is remarkably efficient; 70–80% of the bound (Bpa^8)-SP derivative and 40–50% of the bound (Bpa^4)-SP derivative undergo photoincorporation upon exposure to UV light. Treatment of the photolabeled receptors with endoglycosidase F to remove asparagine-linked carbohydrates increased the mobility of the photolabeled receptors, which are visualized as a discrete radiolabeled band of $M_r = 46,000$.[9] The M_r value for the deglycosylated receptors is in excellent agreement with the molecular mass calculated for the primary sequence of the rat NK-1 receptor.[9]

MAPPING THE PEPTIDE BINDING POCKET

Determination of the site of incorporation of photoaffinity probes such as $[^{125}I]$-(Bpa^8)-SP and $[^{125}I]$-(Bpa^4)-SP identifies directly the region of the receptor that is in contact with a specified position of the peptide. The most straightforward approach to determining the site of insertion of a photoaffinity probe is by fragmentation and sequencing. Prior to preparative scale isolation and sequencing of receptor binding domains, analyses of enzymatic and/or chemical fragmentation procedures conducted on an analytical scale are important for optimization of fragment yield and evaluation of purification steps. Inasmuch as the photoaffinity probes are themselves peptides, a key consideration in the evaluation of potential
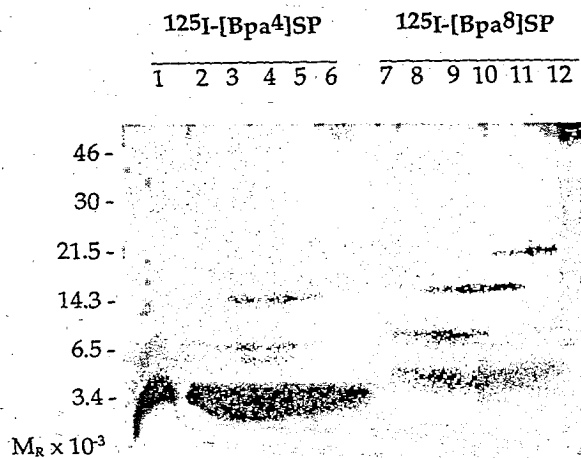
$125I$-$[Bpa^4]SP$ $125I$-$[Bpa^8]SP$

FIGURE 4. Autoradiograph of tryptic fragments of the NK-1 receptor photoaffinity labeled with $[^{125}I]$-(Bpa^4)-SP and $[^{125}I]$-(Bpa^8)-SP. Chinese hamster ovary cells expressing the NK-1 receptor were photoaffinity labeled with either $[^{125}I]$-(Bpa^4)-SP or $[^{125}I]$-(Bpa^8)-SP. Membranes containing labeled NK-1 receptor were prepared from the cells and were incubated for 60 min at 22 °C with trypsin at the following concentrations (mg/mL): 2, 0.6, 0.2, 0.06, 0.02, and 0; lanes 1–6: $[^{125}I]$-(Bpa^4)-SP labeled; lanes 7–12: $[^{125}I]$-(Bpa^8)-SP labeled. The tryptic fragments were resolved by Tricine-SDS-PAGE and visualized by autoradiography.

fragmentation schemes is the requirement that the covalently attached probe remain intact under receptor fragmentation conditions. The results of this type of experiment are shown in FIGURE 4 in which NK-1 receptors expressed in CHO cells were photolabeled with either $[^{125}I]$-(Bpa^8)-SP or $[^{125}I]$-(Bpa^4)-SP and subjected to digestion with increasing concentrations of trypsin and the resulting radiolabeled fragments analyzed by Tricine-SDS-PAGE.

Tryptic digestion of receptor photolabeled with $[^{125}I]$-(Bpa^8)-SP presented a different fragmentation pattern than tryptic digestion of receptor photolabeled with $[^{125}I]$-(Bpa^4)-SP. These results suggest that different amino acid residues serve as the site of covalent attachment for each photoaffinity probe. The experiments further suggest that by this approach it should be possible to generate sufficient quantities of

labeled fragments so that amino acid attachment sites can be identified by purification and microsequencing. Extending this approach to additional substance P derivatives should provide detailed information on the amino acid residues that comprise the peptide binding pocket and thus provide an understanding at the molecular level of peptide-receptor interaction.

REFERENCES

1. NAKANISHI, S. 1991. Mammalian tachykinin receptor. Annu. Rev. Neurosci. **14:** 123–136.
2. INGI, T., Y. KITAJIMA, Y. MINAMITAKE & S. NAKANISHI. 1991. Characterization of ligand-binding properties and selectivities of three rat tachykinin receptors by transfection and functional expression of their cloned cDNAs in mammalian cells. J. Pharmacol. Exp. Ther. **259:** 969–975.
3. BOYD, N. D., C. F. WHITE, R. CERPA, E. T. KAISER & S. E. LEEMAN. 1991. Photoaffinity labeling the substance P receptor using a derivative of substance P containing *p*-benzoylphenylalanine. Biochemistry **30:** 336–342.
4. BOYD, N. D., S. G. MACDONALD, R. KAGE, J. LUBER-NAROD & S. E. LEEMAN. 1991. Substance P receptor, biochemical characterization and interactions with G proteins. Ann. N.Y. Acad. Sci. **632:** 79–93.
5. KAUER, J. C., S. ERICKSON-VIITANEN, H. R. WOLFE, JR. & W. R. DEGRADO. 1986. *p*-Benzoyl-L-phenylalanine, a new photoreactive amino acid. J. Biol. Chem. **261:** 10695–10700.
6. BRESLOW, R. 1980. Acc. Chem. Res. **13:** 170–177.
7. TURRO, N. 1978. Modern Molecular Photochemistry.: 372–377. Benjamin/Cummings. Menlo Park, CA.
8. CASCIERI, M. A., R.-R. C. HUANG, T. M. FONG, A. H. CHEUNG, S. SADOWSKI, E. BER & C. D. STRADER. 1992. Determination of the amino acid residues in substance P conferring selectivity and specificity for the rat neurokinin receptors. Mol. Pharmacol. **41:** 1096–1099.
9. KAGE, R., A. D. HERSHEY, J. E. KRAUSE, N. D. BOYD & S. E. LEEMAN. 1995. Characterization of the substance P (NK-1) receptor in tunicamycin-treated transfected cells using a photoaffinity analog of substance P. J. Neurochem. **64:** 316–321.